

Fas and Fas ligand: *lpr* and *gld* mutations

Shigekazu Nagata and Takashi Suda

*Fas ligand (FasL) is a death factor that binds to its receptor, Fas, and induces apoptosis. Two mutations that accelerate autoimmune disease, *lpr* and *gld*, are known to correspond to mutations within genes encoding Fas and FasL, respectively. Here, Shigekazu Nagata and Takashi Suda summarize current knowledge of Fas and FasL, and discuss the physiological role of the Fas system in T-cell development, cytotoxicity and cytotoxic T lymphocyte (CTL)-mediated autoimmune disease.*

Monoclonal antibodies (mAbs) raised against the Fas (APO-1) cell-surface protein have cell-killing activity^{1,2}. Molecular cloning of Fas cDNA has shown that Fas is a type I membrane protein belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family³⁻⁶. Mouse cell lines expressing human Fas have been established, and crosslinking with antibody against human Fas induces apoptosis of these cells. From these results, it was concluded that Fas transduces an apoptotic signal into cells, and that anti-Fas antibody works as an agonist for the Fas protein. Northern blot hybridization of mouse tissues have indicated that Fas mRNA is abundantly expressed in the thymus, liver, heart, lung, kidney and ovary⁴, but is weakly expressed in various other tissues (T. Suda and S. Nagata, unpublished).

The structure of Fas suggested that it might be a receptor for an unknown cytokine. Progress towards identifying such a ligand was accelerated by the demonstration of a cytotoxic T lymphocyte (CTL) hybridoma (PC60-d10S; d10S), generated by fusing a mouse CTL cell line with a rat lymphoma, that could kill Fas⁺ cells but not Fas⁻ cells⁷. This phenomenon was analyzed further using soluble forms of Fas (Fas-Fc) and the TNF receptor (TNFR-Fc), which were created by fusing the extracellular regions of these molecules to the Fc region of human IgG (Ref. 8). It was found that Fas-Fc, but not TNFR-Fc, inhibited the CTL activity of d10S cells, indicating that these cells express the ligand for Fas (FasL), and that it plays a major role in d10S-mediated cytotoxicity. Indeed, subsequent fluorescence-activated cell sorting (FACS) analysis of d10S cells using biotinylated Fas-Fc has indicated that they express a molecule that binds to Fas (Ref. 8). Furthermore, a subline of d10S (d10S16) that expresses approximately 100-fold more FasL than the original cell line was established by repeated FACS sorting. In concordance with the increased expression of FasL, the d10S16 cells also showed approximately 100-fold more CTL activity against Fas⁺ cells than did the original d10S parent line⁸.

Rat FasL has been purified to homogeneity as a protein of approximately 40 kDa from the solubilized membrane fraction of the d10S subline⁸. The corresponding cDNA was cloned by expression cloning from the d10S subline⁹, and its mouse and human homologs were subsequently cloned by crosshybridization^{10,11}. FasL

has no signal sequence at the N-terminus, but has a domain of hydrophobic amino acids in the center of the molecule, indicating that it is a type II membrane protein with the C-terminal region oriented on the extracellular side of the cell membrane. Mouse and human FasL have 76.9% identity at the amino acid sequence level, and do not demonstrate species specificity¹¹. Approximately 150 amino acids in the extracellular region of FasL have significant homology with members of the TNF family. Recombinant FasL expressed on the surface of COS cells can induce apoptosis in Fas-expressing cells, indicating that FasL is a death factor and that Fas is its receptor.

lpr and *gld*

While establishing a mouse MRL strain, Andrews *et al.*¹² discovered a mouse mutant that develops lymphadenopathy and splenomegaly. The autosomal recessive mutation responsible was located to mouse chromosome 19 (Ref. 13) and is referred to as *lpr* (for lymphoproliferation). Later, Roths *et al.*¹⁴ found a different mutant with a phenotype similar to *lpr*, and this mutation was designated *gld* (for generalized lymphoproliferative disease). The latter is also an autosomal recessive mutation and has been mapped close to the gene for antithrombin III (AT-III) on chromosome 1 (Ref. 15). Meanwhile, another allelic mutation in the *lpr* locus was discovered in the CBA/K1Jms mouse strain¹⁶. Unlike the original *lpr* mutant, this heterozygous defect results in a weak phenotype in mice that are also heterozygous for *gld*. The mutation was therefore designated as *lpr*⁺ (for *lpr* complementing *gld*).

MRL/*lpr* or MRL/*gld* mice develop lymphadenopathy and splenomegaly, and produce large amounts of IgG and IgM antibodies, including anti-DNA antibody and rheumatoid factor autoantibody¹⁷. The mice die of nephritis or arthritis at approximately five months of age. By contrast, other strains of mice carrying *lpr* or *gld* mutations show lymphadenopathy and splenomegaly, but do not develop nephritis or arthritis¹⁸. Since wild-type MRL mice also develop weak and delayed autoimmune disease, it was concluded that *lpr* and *gld* mutations do not induce autoimmune disease, but rather accelerate the underlying predisposition to autoimmune disease.

Lymphocytes that accumulate in the lymph nodes and spleen of *lpr* or *gld* mice express the T-cell marker

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Monoclonal antibodies (mAbs) raised against the Fas (APO-1) cell-surface protein have cell-killing activity^{1,2}. Molecular cloning of Fas cDNA has shown that Fas is a type I membrane protein belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family³⁻⁶. Mouse cell lines expressing human Fas have been established, and crosslinking with antibody against human Fas induces apoptosis of these cells. From these results, it was concluded that Fas transduces an apoptotic signal into cells, and that anti-Fas antibody works as an agonist for the Fas protein. Northern blot hybridization of mouse tissues have indicated that Fas mRNA is abundantly expressed in the thymus, liver, heart, lung, kidney and ovary⁴, but is weakly expressed in various other tissues (T. Suda and S. Nagata, unpublished).

The structure of Fas suggested that it might be a receptor for an unknown cytokine. Progress towards identifying such a ligand was accelerated by the demonstration of a cytotoxic T lymphocyte (CTL) hybridoma (PC60-d105; d105), generated by fusing a mouse CTL cell line with a rat lymphoma, that could kill Fas⁺ cells but not Fas⁻ cells⁷. This phenomenon was analyzed further using soluble forms of Fas (Fas-Fc) and the TNF receptor (TNFR-Fc), which were created by fusing the extracellular regions of these molecules to the Fc region of human IgG (Ref. 8). It was found that Fas-Fc, but not TNFR-Fc, inhibited the CTL activity of d105 cells, indicating that these cells express the ligand for Fas (FasL), and that it plays a major role in d105-mediated cytotoxicity. Indeed, subsequent fluorescence-activated cell sorting (FACS) analysis of d105 cells using biotinylated Fas-Fc has indicated that they express a molecule that binds to Fas (Ref. 8). Furthermore, a subline of d105 (d10516) that expresses approximately 100-fold more FasL than the original cell line was established by repeated FACS sorting. In concordance with the increased expression of FasL, the d10516 cells also showed approximately 100-fold more CTL activity against Fas⁺ cells than did the original d105 parent line⁸.

Rat FasL has been purified to homogeneity as a protein of approximately 40 kDa from the solubilized membrane fraction of the d105 subline⁹. The corresponding cDNA was cloned by expression cloning from the d105 subline⁹, and its mouse and human homologs were subsequently cloned by crosshybridization^{10,11}. FasL

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MRL/*lpr* or MRL/*gld* mice develop lymphadenopathy and splenomegaly, and produce large amounts of IgG and IgM antibodies, including anti-DNA antibody and rheumatoid factor autoantibody¹⁷. The mice die of nephritis or arthritis at approximately five months of age. By contrast, other strains of mice carrying *lpr* or *gld* mutations show lymphadenopathy and splenomegaly, but do not develop nephritis or arthritis¹⁸. Since wild-type MRL mice also develop weak and delayed autoimmune disease, it was concluded that *lpr* and *gld* mutations do not induce autoimmune disease, but rather accelerate the underlying predisposition to autoimmune disease.

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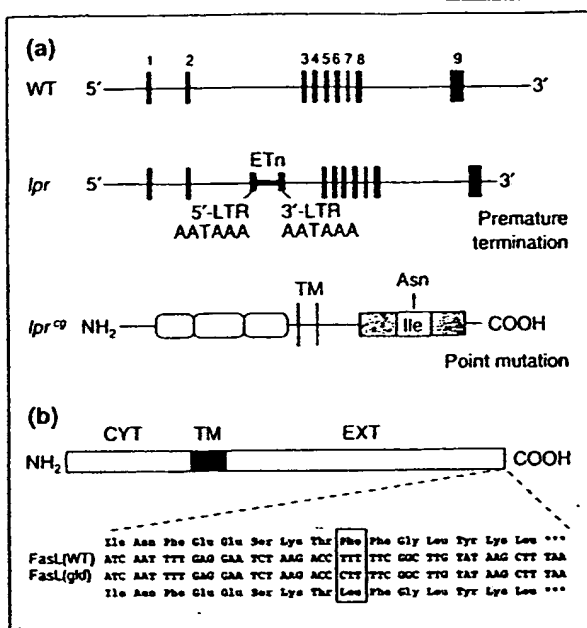


Fig. 1. Mutations of genes encoding Fas and Fas ligand (FasL) in *lpr* and *gld* mice. (a) Structure of the wild-type (WT) gene encoding Fas, and its mutation in *lpr* and *lpr^{ts}* mice. The *lpr* mutation results from the insertion of an early transposable element (ETn), carrying two poly(A) adenylation sites, into intron 2 of the gene encoding Fas. The *lpr^{ts}* mutation results from a point mutation that changes isoleucine to asparagine in the cytoplasmic region of the Fas protein. The shaded area in the cytoplasmic region of Fas denotes the region with similarity to the tumor necrosis factor receptor 1 (Ref. 3), and the boxed extracellular regions indicate protein domains that are related to each other. (b) Structure of FasL and its point mutation in *gld* mice. The structure of the FasL protein is shown schematically. In the sequence below, the boxed region indicates the point mutation of FasL in *gld* mice. Abbreviations: LTR, long terminal repeat; TM, transmembrane region; CYT, cytoplasmic region; EXT, extracellular region.

Thy-1, and the B-cell marker B220 (Ref. 17). These cells also express a rearranged T-cell receptor (TCR), but not a rearranged IgG gene. Furthermore, since neonatal thymectomy prevents the accumulation of these lymphocytes, it appears that they are of T-lineage origin. However, the CD4 and CD8 antigens, which are usually expressed in mature T cells, are not expressed in the lymphocytes that accumulate in *lpr* or *gld* mice. The gene loci encoding CD4 and CD8 are hypomethylated in these lymphocytes, and administration of anti-Thy-1 or anti-CD8 antibody inhibits the development of lymphadenopathy¹⁷. It is likely that these T cells are produced from mature single-positive CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells by suppressing the expression of the CD4 or CD8 antigen, although the possibility that they are produced from double-positive thymocytes by simultaneously downmodulating CD4 and CD8 cannot be ruled out.

Mutations of Fas and FasL in *lpr* and *gld*

As described above, although *lpr* and *gld* are non-allelic mutations, they show the similar phenotypes of lymphadenopathy and splenomegaly. Allen *et al.*¹⁹ have performed a series of bone marrow transplan-

tations among *lpr*, *gld* and wild-type mice in order to establish the relationship between these defects. They concluded that *lpr* and *gld* are mutations in a pair of molecules and, more specifically, that *gld* is a mutation of a soluble or membrane-bound cytokine, while *lpr* is a mutation of its receptor. Considerable data are now available to show that *lpr* and *gld* are mutations in Fas and FasL, respectively.

The gene encoding Fas maps to a location near the *lpr* locus of mouse chromosome 19 (Ref. 4), and northern blot hybridization indicates that *lpr* mice express very little Fas mRNA in the liver and thymus²⁰. Isolation of the germline gene encoding Fas has shown that the wild-type gene is more than 70 kb in length, and is split by eight introns (R. Watanabe-Fukunaga and S. Nagata, unpublished), whereas the mutated gene in *lpr* mice has an early transposable element (ETn), similar to an endogenous retrovirus, inserted into intron 2 (Fig. 1a) (Ref. 21). Since the ETn carries poly(A) adenylation signals (AATAAA) on the long terminal repeat (LTR) sequences, it was assumed that the Fas transcript starting from exon 1 prematurely terminates in intron 2, and is aberrantly spliced. Indeed, small mRNAs coding for exons 1 and 2 have been found in the thymus and liver of *lpr* mice. Furthermore, when the ETn was inserted into the intron of a mammalian expression vector, the expression efficiency of the vector was reduced to a fraction of that of the original. These results suggest that transcription of the gene encoding Fas is impaired in *lpr* mice by the insertion of the transposable element into the intron of the gene. However, inhibition of expression is not complete, as demonstrated by the presence of full-length Fas mRNA, albeit at a low level, in the thymus and liver of the *lpr* mouse, indicating that *lpr* is not a null mutation, but is leaky²².

Unlike the *lpr* strain, *lpr^u* mice express full-length Fas mRNA as abundantly as wild-type mice¹⁰. However, the mRNA carries a point mutation (T to A) in the middle of the Fas cytoplasmic region. This mutation results in an amino acid change, from isoleucine to asparagine (Fig. 1a), and abolishes the ability of Fas to transduce the apoptotic signal into cells.

Recently, the murine gene for FasL was mapped close to the *AT-III* gene on chromosome 1 (Ref. 10), where the *gld* mutation is localized. It has been shown that the *gld* mutation is a point mutation (T to C) near the C-terminus of the coding region for FasL (Fig. 1b) (Ref. 10). This mutation results in the replacement of phenylalanine with leucine, and abolishes the ability of FasL to bind to Fas.

Physiological role of Fas and FasL

T-cell development

The function of Fas and FasL is suggested by the following facts, summarized from findings described above: (1) FasL binds to Fas and induces apoptosis; (2) *lpr* and *gld* mutations result in the development of lymphadenopathy and splenomegaly; and (3) *lpr* and *gld* are loss-of-function mutations in Fas and FasL, respectively (Fig. 1) (Refs 6,10,20). Thus, it appears that the Fas and FasL system is involved in the apoptotic process that occurs during the maturation of T cells.

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Precursor T cells originate in the bone marrow and migrate into the thymus, where they mature into single-positive CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells. During this process, the precursor T cells die by apoptosis at several stages. Those precursor T cells expressing a TCR that can interact with self-major histocompatibility complex (MHC) expressed on thymic epithelium are selected (positive selection), while those that cannot interact with self-MHC die by apoptosis²³. By contrast, precursor T cells that strongly interact with self-antigen complexed with MHC are killed by negative selection²⁴. More than 95% of the precursor T cells that migrate into the thymus die in the thymus, and only about 5% finally emigrate into the periphery as mature T cells^{25,26}. Even in the periphery, mature T cells undergo selection: those that recognize self-antigen are first activated, then die by apoptosis (peripheral clonal deletion)²⁷.

Various studies have examined the steps of T-cell development at which *lpr* or *gld* mice have defects. Although a few reports have suggested that some have defects in the thymic development of T cells²⁸⁻³⁰, the current consensus is that negative and positive selection are normal in these mice^{31,32}. By contrast, Russell *et al.*³³ have reported that the activation-induced cell death of mature T cells does not occur in *lpr* or *gld* mice *in vitro*^{33,34}. Furthermore, when the superantigen staphylococcal enterotoxin B (SEB) is injected into wild-type mice, mature T cells expressing the SEB-reactive VB8 TCR chain initially proliferate, then die by apoptosis. The latter process could not be seen, or was severely retarded, in *lpr* mice³⁵.

Thus, *in vitro* and *in vivo* results suggest the involvement of the Fas system in the clonal deletion of autoreactive T cells in the periphery. As a mechanism for this process, we and others³⁶ propose the model shown in Fig. 2. Antigen-presenting cells (APCs) express the autoreactive peptide as a complex with MHC on their surface. The peptide-MHC complex interacts with the TCR on autoreactive T cells, which activates the cells and induces the expression of Fas and FasL. Such autoreactive T cells then kill each other through interactions between Fas and FasL. In this model, a single cell expresses Fas and FasL. However, it is also possible that a population of T cells may express only FasL, and thus kill autoreactive cells that express Fas while remaining intact itself.

Cytotoxicity

The Fas system also plays an important role in CTL-mediated cytotoxicity. Since FasL is expressed in various CTL cell lines^{7,37} and in activated splenocytes^{8,38}, it is possible that FasL also functions as an effector of CTL-mediated cytotoxicity. Recently, Kägi *et al.*³⁹ generated mice deficient in the gene encoding perforin, an effector of cytotoxicity^{39,40}. Although most CTL activities are abolished in these mice, some remained, and these residual functions appear to be mediated by FasL (Refs 41,42). Furthermore, since CTLs from perforin-deficient mice show no cytotoxic activity towards Fas⁺ cells^{41,42}, it appears that perforin and FasL are the only effector molecules in CTLs (at least under these short-term assay conditions).

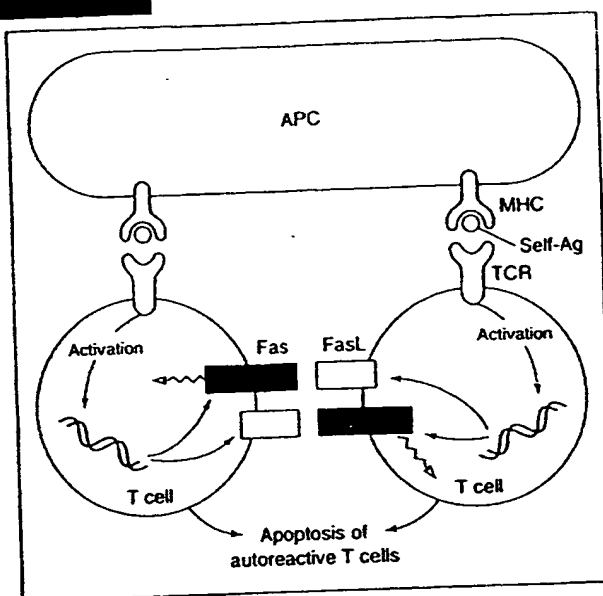


Fig. 2. A model for Fas-mediated clonal deletion in the periphery. Antigen-presenting cells (APCs) express self-antigen (Ag) as a complex with major histocompatibility complex (MHC). The interaction between the MHC-Ag complex and the T-cell receptor (TCR) on autoreactive T cells results in T-cell activation. This induces the expression of genes encoding Fas and Fas ligand (FasL). Subsequent interaction between T cells expressing Fas and FasL results in mutual Fas-mediated apoptosis (zigzagged arrow) and clonal deletion.

Fas outside the lymphoid system

Fas, the receptor for a death factor, is abundantly expressed not only in activated lymphocytes, but also in the liver, heart and ovary⁴. A cytotoxic mAb (Jo2), generated against mouse Fas, has been shown to kill wild-type mice rapidly following intraperitoneal (i.p.) administration, but does not kill *lpr* mice⁴³. The mAb acts by inducing a phenotype similar to fulminant hepatitis. Thus, two hours after injection, the serum levels of glutamic oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) were found to increase dramatically, and reached approximately 200- and 1000-fold over basal level, respectively. Histochemical analysis showed massive hemorrhagic necrosis in the entire liver, and electron microscopy of the affected hepatocytes revealed condensed and fragmented nuclei. Since this morphology is characteristic of apoptosis, it may be concluded that the anti-Fas antibody was causing the hepatocytes to die. Furthermore, since cell death occurred so rapidly and widely, neutrophils and macrophages could not phagocytose the apoptotic cells. This resulted in the progression of hepatocytes into secondary necrosis, releasing lethal, toxic components.

A mouse model system for human fulminant hepatitis has been developed^{44,45} using transgenic mice carrying the human hepatitis B virus (HBV) on an albumin regulatory promoter. When CTLs specific for the HBV antigen were injected into the transgenic mice, they induced apoptosis in the liver, resulting in fulminant hepatitis. Since FasL is expressed in the activated CTLs, we propose a model for the involvement of the Fas system in fulminant hepatitis as shown in Fig. 3.

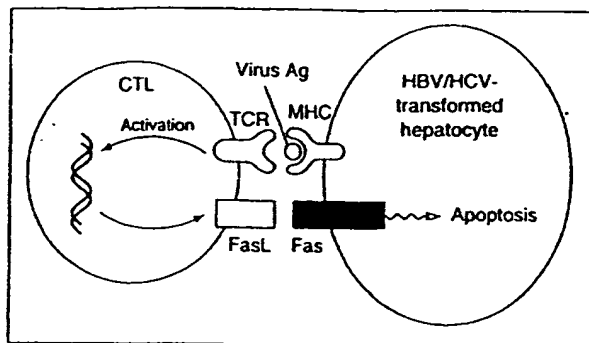


Fig. 3. A proposed mechanism for Fas-mediated fulminant hepatitis. Hepatocytes transformed with human hepatitis B virus (HBV) or C virus (HCV) express the virus antigen as a complex with major histocompatibility complex (MHC). Interaction between the MHC-viral Ag complex and the T-cell receptor (TCR) on cytotoxic T lymphocytes (CTLs) activates the CTL, and induces the expression of the gene encoding Fas ligand (FasL). FasL expressed on the cell surface of the CTL then binds to Fas on hepatocytes and induces their apoptosis.

Hepatocytes transformed by HBV or hepatitis C virus (HCV) would express virus antigen on their surface as a complex with MHC. The interaction of viral peptide with the TCR activates the CTLs, and induces the expression of the gene encoding FasL. FasL then binds to Fas expressed on hepatocytes and induces apoptosis.

Conclusions

Studies using an anti-Fas antibody with cytolytic activity have led us to propose a model for the FasL 'death factor' and its receptor. The discovery that the murine *lpr* and *gld* defects correspond to mutations in the genes encoding Fas and FasL, respectively, suggests that autoimmune disease can be mediated (or accelerated) by defects in apoptosis genes. It is possible that such mutations also cause autoimmune disease in humans.

Furthermore, human fulminant hepatitis may be caused by abnormal activation of FasL expressed on CTLs. Since CTL-mediated autoimmune disease also occurs in other pathologies, such as Graves' disease and chronic thyroiditis⁴⁶, the involvement of the Fas system in these diseases is worthy of investigation. If the involvement of the Fas-FasL pair in these diseases is confirmed, the soluble form of Fas, as well as neutralizing anti-Fas or anti-FasL antibodies, could prove clinically useful.

We thank O. Hayaishi and C. Weissmann for encouragement and discussion. The work was carried out in collaboration with M. Adachi, J. Ogasawara, T. Takahashi, M. Tanaka, T. Kondo, M. Enari, H. Hug, N. Itoh and R. Watanabe-Fukunaga, and supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan. We also thank K. Mimura for secretarial assistance.

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Combinatorial libraries: new insights into human organ-specific autoantibodies

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The recent application of immunoglobulin (Ig) gene combinatorial library technology has led to a logarithmic increase in information concerning human, disease-associated, organ-specific autoantibodies of the IgG class. As reviewed here by Basil Rapoport, Stefano Portolano and Sandra McLachlan, the molecular cloning, analysis and expression of the genes for increasing numbers of these human, monoclonal autoantibodies is providing new insight into the genetic background and epitopic repertoires of such molecules.

Human autoimmune disease may be organ specific or systemic. The most common organ-specific autoimmune diseases in humans affect the thyroid gland and the pancreatic β -cell. The prototypes of systemic autoimmunity are the connective tissue disorders, lupus erythematosus, scleroderma and rheumatoid arthritis. A second classification of human autoimmunity relates to whether the pathogenesis of the disease is considered to involve primarily a humoral or a cell-mediated effector mechanism. The humoral component predominates in autoimmune responses against, for example, the skeletal muscle endplate in myasthenia gravis (MG) and the thyroid in Graves' disease and Hashimoto's thyroiditis. By contrast, autoimmune diseases such as multiple sclerosis are considered to be mediated primarily by the cellular immune response. Insulin-dependent diabetes mellitus (IDDM) is included in this cell-mediated category, although recent data suggest that the humoral response may be more important than previously considered¹.

Autoantibody generation, at least of the IgG class, is dependent on T cells. Nevertheless, dissecting out the components of the humoral autoimmune response is an essential prerequisite for determining the role of antigen-specific (not simply tissue-infiltrating) T cells involved in specific autoantibody generation. For this

purpose, the information required includes: (1) identification of the primary autoantigens in the disease (this has already been accomplished in many autoimmune diseases); (2) determining the repertoire of autoantibodies to a specific autoantigen; (3) mapping the autoantigenic epitopes; (4) analyzing the immunoglobulin (Ig) variable (V)-region genes coding for these autoantibodies; and (5) ultimately determining the role of B cells in presenting autoantigen to T cells.

Why clone human organ-specific autoantibodies?

Although some patients with Graves' disease or MG show oligoclonal serum autoantibodies, most organ-specific autoantibodies are polyclonal. Therefore, the generation of monoclonal antibodies (mAbs) is essential for dissecting the epitopes recognized by the polyclonal autoantibodies. Indeed, there has been no difficulty in generating murine mAbs by immunizing animals with antigens such as those associated with human autoimmune thyroid disease or MG.

However, despite the ease with which mAbs directed to human autoantigens can be generated in mice, their value is limited by a number of factors. Autoantigens in organ-specific autoimmunity are generally large proteins with complex, nonrepetitive, three-dimensional structures; examples include thyroid peroxidase (TPO),

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